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(54) Title: LIPASE VARIANTS

(57) Abstract

Lipases comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule are mutated so as to substitute a non-aromatic amino acid residue of a lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues may participate in the interaction with the substrate at or during hydrolysis, with an aromatic amino acid residue.

LIPASE VARIANTS

FIELD OF INVENTION

The present invention relates to novel lipase enzyme variants with improved properties, DNA constructs coding for the expression of said variants, host cells capable of expressing the variants from the DNA constructs, as well as a method of producing the variants by cultivating said host cells.

BACKGROUND OF THE INVENTION

The advent and development of recombinant DNA techniques has had a profound influence on the field of protein chemistry. It has been envisaged that these techniques will make it possible to design peptides and proteins, such as enzymes, in accordance with specific criteria, thus permitting the production of compounds with desired properties.

Due to the availability of such techniques, it has become possible to construct enzymes with desired amino acid sequences, and a fair amount of research has been devoted to this object.

The primary structure of a number of lipases has been determined and described in the literature (Boel et al., *Lipids* **23**, 701-706 (1988), de Caro et al., *Biochim. Biophys. Acta* **671**, 129-138 (1981), Winkler et al., *Nature* **343**, 771-774 (1990)). Furthermore also the tertiary structure of a more limited number of lipases has been elucidated (Winkler et al., *Nature* **343**, 771-774 (1990), Brady et al., *Nature* **343**, 767-770 (1990) J.D. Schrag et al., Nature 351, 1991, pp. 761-764). From these investigations it appears that lipases seem to have certain structural features in common, but that,on the other hand, major structural variations also exist among the lipases.

WO 92/05249 discloses lipase variants with improved properties, in which certain characteristics of wild-type lipase enzymes have been changed by specific modifications of their amino acid sequences. For instance, the electrostatic charge and/or hydrophobicity of a so-called lipid contact zone of the wild-type lipase enzymes have been changed, and the accessibility of lipid substrate to the active site has been improved, mainly by substituting or deleting amino acid residues present in the native lipase molecule.

SUMMARY OF THE INVENTION

The present inventors have now surprisingly identified further amino acid modifications leading to the construction of novel lipase variants having improved properties.

Accordingly, in one aspect, the present invention relates to a lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, in which a non-aromatic amino acid residue of a lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues may participate in the interaction with the substrate at or during hydrolysis, has been substituted with an aromatic amino acid residue. In the following disclosure this type of lipase variant is termed lipase variant I.

In the present context, the term "trypsin-like" is intended to indicate that the parent lipase comprises a catalytic triad at the active site corresponding to that of trypsin, i.e. the amino acids Ser, His and one of Asp, Glu, Asn or Gln. Some lipases may also comprise a surface loop structure which covers the active serine when the lipase is in inactive form (an example of such a lipase is described by Brady et al., Nature 343, 1990, pp. 767-770). When the lipase is activated, the loop structure is shifted to expose the active site residues, creating a sur-

face with increased surface hydrophobicity which interacts with the lipid substrate at or during hydrolysis. For the present purpose, this surface is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface (or a corresponding surface of lipases which do not comprise such a loop structure). The amino acid residues, optionally in the form of loop structures, may participate in lipase interaction with the substrate at or during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface. During hydrolysis of the triglycerides, fatty acids and mono- and di-glycerides are formed in varying amounts.

The lipid contact zone of the *Humicola lanuginosa* lipase discussed in detail in the present application is defined by amino acid residues 21-25, 36-38, 56-62, 81-98, 110-116, 144-147, 172-174, 199-213 and 248-269. These residues have been identified on the basis of computer model simulations of the interaction between the lipase and a lipid substrate.

In the present context "an aromatic amino acid residue" is intended to mean a residue of tyrosine, tryptophan or phenylalanine, and the term "non-aromatic amino acid residue" is intended to include a residue of an amino acid different from tyrosine, tryptophan and phenylalanine.

In a further aspect the invention relates to specific lipase variants in which one or more amino acid residues in specific positions of the *Humicola lanuginosa* lipase disclosed in WO 92/05249, the cDNA and amino acid sequence of which are shown in SEQ ID Nos. 1 and 2, or in similar positions of lipases of other origins has/have been substituted with other amino acid residues. These variants are further discussed below.

The present invention also relates to a DNA construct comprising a DNA sequence encoding a lipase variant as indicated above, a recombinant expression vector carrying said DNA construct, a cell transformed with the DNA construct or the ex-

pression vector, as well as a method of producing a lipase variant of the invention by culturing said cell under conditions conducive to the production of the lipase variant, after which the lipase variant is recovered from the culture.

The invention further relates to a detergent additive comprising a lipase variant of the invention, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme, as well as to a detergent composition comprising lipase variant of the invention.

DETAILED DISCLOSURE OF THE INVENTION

In describing lipase variants according to the invention, the following nomenclature is used for ease of reference:
Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of aspartic acid for tryptophan in position 96 is shown as:

Asp 96 Trp or D96W

Multiple mutations are separated by pluses, i.e.:

Asp 96 Leu + Leu 206 Val or D96L+L206V

representing mutations in positions 96 and 206 substituting aspartic acid and leucine for leucine and valine, respectively. The lipase variants are mostly defined by use of the conventional one-letter amino acid code.

According to the invention, lipase variant I is preferably one in which the non-aromatic amino acid residue to be substituted is a glutamic acid or an aspartic acid residue, and preferably one located in position 96 of the amino acid sequence of the mature *H. lanuginosa* lipase shown in SEQ ID No. 2, or in a similar position of a parent lipase of another origin as discussed in further detail below.

Specific lipase variants of the invention prepared from said *H. lanuginosa* lipase comprises one or more amino acid residues substituted as follows:

E56H, P, M, W, Y, F, I, G, C, V;
D96H, E, P, M, W, Y, F, I, G, C, V;
L259N, D, C, Q, E, H, I, M, F, P, W, Y;
L206K,R, N, D, C, Q, E, H, I, M, F, P, W, Y

A particularly interesting effect may also be obtained when the lipase variant of the invention comprises more than one substitution, preferably two substitutions. For instance, the following variants of the *H. lanuginosa* lipase have been found to be of interest:

D96W+E210N;
D254K+L259I;
D96L+L206V;
D96L+L206S;
D96W+D102N;
D96L+L259I+L206V;
E56Q+L259I+L206V.

Lipase variants prepared from lipases of other origins by similar substitutions as those described above for the *H. lanuginosa* lipase are also considered to be within the scope of the present invention.

In the present context, the term "similar substitutions" is intended to indicate amino acid substitutions of other lipases, which are performed in similar positions to those identified above for the *H. lanuginosa* lipase. Similar positions may be identified on the basis of a comparison of the three-dimensional structure of the lipase in question with that of the *H. lanuginosa* lipase. The three-dimensional structure of the *H. lanuginosa* lipase is shown in Fig. 1A and 1B of WO 92/05249, and the three-dimensional structure of the parent lipase to be modified may either be known or elucidated by conventional

methods, e.g. involving X-ray analysis. The amino acid residues to be substituted and the ones to be inserted preferably belong to the same type of amino acid (e.g. hydrophobic, hydrophillic, etc.), but need not be identical with the actual amino acid residue of the *H. lanuginosa* lipase.

Although the parent lipase may be derived from a variety of sources such as mammalian lipases, e.g. pancreatic, gastric, hepatic or lipoprotein lipases, it is generally preferred that it is a microbial lipase. As such, the parent lipase may be selected from yeast, e.g. *Candida*, lipases, bacterial, e.g. Pseudomonas, lipases or fungal, e.g. *Humicola* or *Rhizomucor* lipases. It is particularly preferred to select the parent lipase from a group of structurally homologous lipases. For instance, the parent lipase may be a *Rhizomucor miehei* lipase, in particular the lipase described in EP 238 023, or, as mentioned above, a *H. lanuginosa* lipase.

It should be noted that the *H. lanuginosa* lipase and the *Rhizomucor miehei* lipase belong to the same group of lipases. This implies that the overall three-dimensional structure of the two lipases is very similar and has been shown by X-ray crystallography to be highly homologous (a computer model of the *H. lanuginosa* and the *Rh. miehei* lipase is shown in Figs. 1A and B and 2A and B, respectively, of WO 92/05249 from which the similarities between the lipid contact zones of the two lipases are clearly apparent). It is therefore probable that modifications of the type indicated for the *H. lanuginosa* lipase will also be functional for the *Rh. miehei* lipase.

It should be noted that, according to the invention, any one of the modifications of the amino acid sequence indicated above may be combined with any of the other modifications described herein or anyone of the modifications mentioned in WO 92/05249.

The lipase variants of the invention may be prepared by isolating a DNA sequence encoding a parent lipase, suitable modifying said sequence, e.g. by site-directed mutagenesis, to

encode for the variant in question and subsequently introducing the modified DNA sequence into a suitable host organism capable of expressing the variant in question. The DNA sequence of the DNA construct of the invention may be a cDNA, genomic DNA or synthetic DNA sequence or any combination of such sequences obtained in accordance with conventional technology. Suitable techniques for cloning and mutating DNA sequences are disclosed in detail in WO 92/05249, the content of which is hereby incorporated by reference. The techniques are further exemplified in the following examples.

The expression of lipase variants of the invention may be obtained as follows. A mutated lipase-coding sequence produced, e.g. as described in WO 92/04249, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the lipase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant lipase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

According to one embodiment a cell of the genus *Bacillus*, such as *B. licheniformis*, *B. lentus*, or *B. subtilis* is transformed by an expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as *B. subtilis* a signal sequence may follow the translation initia-

tion signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when is present.

In a currently preferred method of producing lipase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of *Aspergillus* sp., such as *A. niger*, *A. nidulans* or *A. oryzae*. The use of *A. oryzae* in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of lipase variants in *Aspergillus*, the DNA sequence coding for the lipase variant is preceded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in *Aspergillus* and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease or *A. oryzae* triose phosphate isomerase.

In particular when the host organism is *A. oryzae*, a preferred promoter for use in the process of the present invention is the *A. oryzae* TAKA amylase promoter as it exhibits a strong transcriptional activity in *A. oryzae*. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

To ensure secretion of the lipase variant from the host cell, the DNA sequence encoding the lipase variant may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease, or a gene encoding a *Humicola* cellulase, xylanase or lipase. The signal sequence is preferably derived from the gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable α-amylase or *A. niger* glucoamylase.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing *Aspergillus* cells. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Detergent Compositions

According to the invention, the lipase variant may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may

optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000, ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50 % of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid or soap. It may also contain 0-40 % of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as an amylase, a lipase, a cutinase, a protease, a cellulase, a peroxidase or an oxidase.

The detergent may contain 1-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative as e.g. an aromatic borate ester, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
- alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
- alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
- sodium carbonate (as Na_2CO_3)	14 - 20%
- soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
- zeolite (as $NaAlSiO_4$)	15 - 22%
- sodium sulfate (as Na_2SO_4)	0 - 6%
- sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
- sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
- TAED	2 - 6%
- carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
- alcohol ethoxysulfate	

(e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 3%
- alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
- sodium carbonate (as Na_2CO_3)	15 - 21%
- soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
- zeolite (as $NaAlSiO_4$)	24 - 34%
- sodium sulfate (as Na_2SO_4)	4 - 10%
- sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
- carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
- soap as fatty acid (e.g. C_{16-22})	1 - 3%
- sodium carbonate (as Na_2CO_3)	10 - 17%
- soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
- zeolite (as $NaAlSiO_4$)	23 - 33%
- sodium sulfate (as Na_2SO_4)	0 - 4%
- sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
- TAED	2 - 8%
- phosphonate (e.g. EDTMPA)	0 - 1%
- carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer,	

PVP, PEG)	1 - 3%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
- sodium carbonate (as Na_2CO_3)	14 - 22%
- soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
- zeolite (as $NaAlSiO_4$)	25 - 35%
- sodium sulfate (as Na_2SO_4)	0 - 10%
- carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
- soap as fatty acid (e.g. oleic acid)	3 - 13%
- alkenylsuccinic acid (C_{12-14})	0 - 13%
- aminoethanol	8 - 18%
- citric acid	2 - 8%
- phosphonate	0 - 3%

- polymers (e.g. PVP, PEG)	0 - 3%
- borate (as B_4O_7)	0 - 2%
- ethanol	0 - 3%
- propylene glycol	8 - 14%
- enzymes	0 - 5%
- minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	3 - 9%
- soap as fatty acid (e.g. oleic acid)	3 - 10%
- zeolite (as $NaAlSiO_4$)	14 - 22%
- potassium citrate	9 - 18%
- borate (as B_4O_7)	0 - 2%
- carboxymethylcellulose	0 - 2%
- polymers (e.g PEG, PVP)	0 - 3%
- anchoring polymers as e.g. lauryl metharylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
- glycerol	0 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- fatty alcohol sulfate	5 - 10%
- ethoxylated fatty acid monoethanolamide	3 - 9%

- soap as fatty acid	0 - 3%
- sodium carbonate (as Na_2CO_3)	5 - 10%
- soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
- zeolite (as $NaAlSiO_4$)	20 - 40%
- sodium sulfate (as Na_2SO_4)	2 - 8%
- sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
- TAED	2 - 7%
- polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

- linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
- ethoxylated fatty acid monoethanolamide	5 - 11%
- soap as fatty acid	0 - 3%
- sodium carbonate (as Na_2CO_3)	4 - 10%
- soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
- zeolite (as $NaAlSiO_4$)	30 - 50%
- sodium sulfate (as Na_2SO_4)	3 - 11%
- sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
- polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

- linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
- nonionic surfactant,	1 - 4%

- soap as fatty acid	2 - 6%
- sodium carbonate (as Na_2CO_3)	14 - 22%
- zeolite (as $NaAlSiO_4$)	18 - 32%
- sodium sulfate (as Na_2SO_4)	5 - 20%
- sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
- sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
- bleach activator (e.g. NOBS or TAED)	1 - 5%
- carboxymethylcellulose	0 - 2%
- polymers (e.g. polycarboxylate or PEG)	1 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
- alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	3 - 9%
- soap as fatty acid (e.g. lauric acid)	0 - 3%
- aminoethanol	1 - 5%
- sodium citrate	5 - 10%
- hydrotrope (e.g. sodium toluenesulfonate)	2 - 6%
- borate (as B_4O_7)	0 - 2%
- carboxymethylcellulose	0 - 1%
- ethanol	1 - 3%
- propylene glycol	2 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
- alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	6 - 12%
- aminoethanol	2 - 6%
- citric acid	8 - 14%
- borate (as B_4O_7)	1 - 3%
- polymer (e.g. maleic/acrylic acid copolymer, anchoring polymers as e.g. lauryl methacrylate/acrylic acid copolymer and CMC)	0 - 3%
- glycerol	3 - 8%
- enzymes	0 - 5%
- minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
- nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
- sodium carbonate (as Na_2CO_3)	8 - 25%
- soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
- sodium sulfate (as Na_2SO_4)	0 - 5%
- zeolite (as $NaAlSiO_4$)	15 - 28%
- sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
- bleach activator (TAED or NOBS)	0 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) where the content of linear alkylbenzenesulfonate - or a part of it - is substituted by alkyl sulfate (C_{12}-C_{18}).

14) Detergent formulations as described in 1) - 13) which contain a stabilized or encapsulated peracid either as an additional component or as a substitute for already specified bleach systems.

15) Detergent compositions as described in 3), 7), 9) and 12) where the content of perborate is substituted with percarbonate.

16) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant as e.g. linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The lipase variant of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipase variant may be added in an amount corresponding to 0.001-100 mg of enzyme per liter of wash liquor.

Dishwashing detergent composition

Furthermore, the lipase variant may be used as an ingredient in dishwashing detergent composition. The dishwashing detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will contain 0-90% of non-ionic surfactant such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-

containing types. The detergent composition usually contains 1-90% of detergent builders.

Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts especially alkali metal pyrophosphates, orthophosphates, polyphosphates, and phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates and silicates as well as the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best-known representatives.

Examples of suitable organic builders include the alkali metal, ammonium and substituted ammonium, citrates, succinates, malonates, fatty acid sulphonates, carboxymetoxy succinates, ammonium polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates, polyacetyl carboxylates and polyhydroxsulphonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as

a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g.propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

The dishwashing detergent composition may also comprise other enzymes, in particular an amylase, a protease and/or a cellulase.

The dishwashing detergent composition of the invention may also contain other conventional detergent ingredients, e.g. deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners and perfumes.

Finally, the variant of the invention may be used in conventional dishwashing detergents, e.g. any of the detergents described in any of the following patent publications:
EP 551670, EP 533239, WO 9303129, EP 507404, US 5141664, GB 2247025, EP 414285, GB 2234980, EP 408278, GB 2228945, GB 2228944, EP 387063, EP 385521, EP 373851, EP 364260, EP 349314, EP 331370, EP 318279, EP 318204, GB 2204319, EP 266904, US 5213706, EP 530870, CA 2006687, EP 481547, EP 337760, WO 93/14183, US 5223179, WO 93/06202, WO 93/05132, WO 92/19707, WO 92/09680, WO 92/08777, WO 92/06161, WO 92/06157, WO 92/06156, WO 91/13959, EP 399752, US 4941988, US 4908148.

Softening composition

Furthermore, the lipase variants of the invention may be used in softening compositions:

The lipase variant may be used in fabric softeners, e.g. as described in Surfactant and Consumer Products, Ed. by J. Falbe, 1987, pp 295-296; Tenside Surfactants Detergents, 30 (1993), 6, pp 394-399; JAOCS, Vol. 61 (1984), 2, pp 367-376; EP 517 762; EP 123 400; WO 92/19714; WO 93/19147; US 5,082,578; EP 494 769; EP 544 493; EP 543 562; US 5,235,082; EP 568 297; EP 570 237.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in the following with reference to the appended drawings, in which

Fig. 1 is a schematic representation of the preparation of plasmids encoding lipase variants by polymerase chain reaction (PCR); and

Fig. 2 is a schematic representation of the three-step mutagenesis by PCR;

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

GENERAL METHODS

Expression of H. lanuginosa lipase in Aspergillus oryzae

Cloning of *H. lanuginosa* lipase is described in EP 305,216. This patent application also describes expression and characterization of the lipase in Aspergillus *oryzae*. The expression plasmid used is termed p960.

The expression plasmid used in this application is identical to p960, except for minor modifications immediately 3' to the li-

pase coding region. The modification was made in the following way: p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from plasmid pBR322, in which the NheI fragment was filled in with Klenow polymerase, was cloned, thereby creating plasmid pAO1 (shown in Fig. 5 of WO 92/05249) which contains unique BamHI and NheI sites. Between these unique sites a BamHI/XbaI fragment from p960 was cloned to give pAHL (shown in Fig. 6 of WO 92/05249).

Site-directed in vitro mutagenesis of a lipase gene

The approach used for introducing mutations into the lipase genes is described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989). It involves the 3-step generation of a PCR (polymerase chain reaction) fragment containing the desired mutation introduced by using a chemically synthesized DNA-strand as one of the primers in the PCR-reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid. This method is thoroughly described in example 3. In figures 1 and 2 the method is further outlined.

EXAMPLES

EXAMPLE 1

Construction of a plasmid expressing the D96W variant of H. lanuginosa lipase

Linearization of plasmid pAHL

The circular plasmid pAHL was linearized with the restriction enzyme SphI in the following 50 μl reaction mixture: 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg plasmid and 2 units of SphI. The digestion was carried out for 2 hours at 37°C. The reaction mixture was extracted with phenol (equilibrated with Tris-HCl, pH 7.5) and precipitated by adding 2 volumes of ice-cold 96% ethanol. After centrifugation

and drying of the pellet, the linearized DNA was dissolved in 50 μl H_2O and the concentration estimated on an agarose gel.

3-step PCR mutagenesis

As shown in Fig. 2, 3-step mutagenisation involves the use of four primers:

Mutagenisation primer (=A):
5'-ATTTATTTCTTTCAACCAGAAGTTAAGATTCCC-3'

PCR Helper 1 (=B):
5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAAATCGGC-3'

PCR Helper 2 (=C): 5'-CCATGGCTTTCACGGTGTCT-3'
PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3'

All 3 steps were carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid was added to a total of 100 μl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were carried out.

The concentration of the PCR product was estimated on an agarose gel. Then, step 2 was carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid was contained in a total of 100 μl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C was carried out.

To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D was added (1 μl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C were

carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment

The product from step 3 was isolated from an agarose gel and re-dissolved in 20 μl H_2O. Then, it was digested with the restriction enzymes BamHI and BstxI in a total volume of 50 μl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT and 10 units of each enzyme. Incubation was at 37°C for 2 hours. The 733 bp BamHI/BstxI fragments as isolated from an agarose gel.

Ligation to expression vector pAHL

The expression plasmid pAHL was cleaved with BamHI and BstxI under conditions indicated above and the large fragment was isolated from an agarose gel. To this vector, the mutated fragment isolated above was ligated and the ligation mix was used to transform E.coli. The presence and orientation of the fragment was verified by cleavage of a plasmid preparation from a transformant with restriction enzymes. Sequence analysis was carried out on the double-stranded plasmid using the di-deoxy chain termination procedure developed by Sanger. The plasmid was named pAHLD96W and is identical to pAHL, except for the altered codon.

EXAMPLE 2

Construction of plasmids expressing other variants of H. lanuginosa lipase

The following mutants were constructed using the same method as described in example 1, except that the restriction enzyme XhoI and BstxI were used for digesting the PCR-product and the vector used for recloning of the mutated fragments for D254K/L259I and L259I. Plasmid names and primers used for the modifications are listed below.

Plasmid name	Primer A sequence
pAHLD96F	5'-ATTTATTTCTTTCAAGAAGAAGTTAAGATTCCC-3'
pAHLD96V	5'-ATTTATTTCTTTCAAAACGAAGTTAAGATTCCC -3'
pAHLL259I	5'-CCGAAGTACCAAATGTGAGCAGGGATATCC-3'
pAHLD254K+L259I	5'-CCGAAGTACCAAATGTGAGCAGGGATCTTCGGAATGTTAGG-3'

EXAMPLE 3

Transformation of Aspergillus oryzae (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) was inoculated with spores of A. oryzae and incubated with shaking for about 24 hours. The mycelium was harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium was suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH = 5.8. The suspension was cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234, batch 1687 was added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) was added and incubation with gentle agitation continued for 1.5 - 2.5 hours at 37°C until a large number of protoplasts was visible in a sample inspected under the microscope.

The suspension was filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation was performed for 15 min. at 1000 g and the protoplasts were collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$) were added to the protoplast suspension and the mixture was centrifugated for 5 min. at 1000 g. The protoplast pellet was resuspended in 3 ml of STC and repelleted. This was repeated. Finally, the protoplasts were resuspended in 0.2 - 1 ml of STC. 100 μl of protoplast suspension was mixed with 5 - 25 μg of p3SR2 (an A. nidulans amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 μl of STC. The mixture was left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10

mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 was added and carefully mixed (twice) and finally 0.85 ml of the same solution was added and carefully mixed. The mixture was left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet was resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts were spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4 - 7 days at 37°C spores were picked, suspended in sterile water and spread for single colonies. This procedure was repeated and spores of a single colony after the second reisolation were stored as a defined transformant.

EXAMPLE 4

Expression of the lipase variant D96W in A. oryzae

pAHLD96W was transformed into A. oryzae IFO 4177 by cotransformation with p3SR2 containing the amdS gene from A. nidulans as described in example 3. Protoplasts prepared as described were incubated with a mixture of equal amounts of pAHLD96W and p3SR2, approximately 5 μg of each were used. 9 transformants which could use acetamide as sole nitrogen source were reisolated twice. After growth on YPD for three days, culture supernatants were analyzed using the assay for lipase activity described in example 5 (Purification of lipase variants of the invention). The best transformant was selected for further studies and grown in a 1 l shake-flask on 200 ml FG4 medium (3% soy meal, 3% maltodextrin, 1% peptone, pH adjusted to 7.0 with 4 M NaOH) for 4 days at 30°C. Under these conditions the transformant gave about 500 lipase units per ml of culture.

The other lipase variants were produced essentially as described above, using the general procedure described in example 3.

EXAMPLE 5

Purification of lipase variants of the invention

Assay for lipase activity :

A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.

Lipase activity was assayed at pH 7 using pH stat method. One unit of lipase activity (LU/mg) was defined as the amount needed to liberate one micromole fatty acid per minute.

Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to 7 and add gradually an equal volume of cold 96 % ethanol. Allow the mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

Step 2:- Ion exchange chromatography. Filter the supernatant and apply on DEAE-fast flow (Pharmacia TM) column equilibrated with 50 mM tris-acetate buffer pH 7. Wash the column with the same buffer till absorption at 280 nm is lower than 0.05 OD. Elute the bound enzymatic activity with linear salt gradient in the same buffer (0 to 0.5 M NaCl) using five column volumes. Pool the fractions containing enzymatic activity .

Step 3:- Hydrophobic chromatography. Adjust the molarity of the pool containing enzymatic activity to 0.8 M by adding solid Ammonium acetate. Apply the enzyme on TSK gel Butyl- Toyopearl 650 C column (available from Tosoh Corporation Japan) which was pre-equilibrated with 0.8 M ammonium acetate. Wash the unbound material with 0.8 M ammonium acetate and elute the bound material with distilled water.

Step 4:- Pool containing lipase activity is diluted with water to adjust conductance to 2 mS and pH to 7. Apply the pool on High performance Q Sepharose (Pharmacia) column pre-equilibrated with 50 mM tris -acetate buffer pH 7. Elute the bound enzyme with linear salt gradient.

EXAMPLE 6

The washing performance of lipase variants of the invention
The washing performance of H. lanuginosa lipase variants of the invention was evaluated on the basis of the enzyme dosage in mg of protein per litre according to OD_{280} compared to the wild-type H. lanuginosa lipase.

Wash trials were carried out in 150 ml beakers placed in a thermostated water bath. The beakers were stirred with triangular magnetic rods.

The experimental conditions were as follows:

Method:	3 cycles with overnight drying between each cycle	
Wash liquor:	100 ml per beaker	
Swatches:	6 swatches (3.5 x 3.5 cm) per beaker	
Fabric:	100% cotton, Test Fabrics style #400	
Stain:	Lard coloured with Sudan red (0.75 mg dye/g of lard). 6 µl of lard heated to 70°C was applied to the centre of each swatch. After application of the stain, the swatches were heated in an oven at 75°C for 30 minutes. The swatches were then stored overnight at room temperature prior to the first wash.	
Detergent:	LAS (Nansa 1169/P, 30% a.m.)	1.17 g/l
	AEO (Dobanol 25-7)	0.15 g/l
	Sodium triphosphate	1.25 g/l
	Sodium sulphate	1.00 g/l
	Sodium carbonate	0.45 g/l
	Sodium silicate	0.15 g/l
pH: 10.2		
Lipase conc.:	0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per litre	
Time:	20 minutes	
Temperature:	30°C	
Rinse:	15 minutes in running tap water	

Drying: overnight at room temperature (~20°C, 30-50% RH)

Evaluation: after the 3rd wash, the reflectance at 460 nm was measured.

Results

Dose-response curves were compared for the lipase variants and the native H. lanuginosa lipase. The dose-response curves were calculated by fitting the measured data to the following equation:

$$\Delta R = \Delta R_{max} \frac{C^{0.5}}{K + C^{0.5}} \qquad (I)$$

where ΔR is the effect expressed in reflectance units
C is the enzyme concentration (mg/l)
ΔR_{max} is a constant expressing the maximum effect
K is a constant; K^2 expresses the enzyme concentration at which half of the maximum effect is obtained.

Based on the characteristic constants ΔR_{max} and K found for each lipase variant as well as the wild-type lipase, improvement factors were calculated. The improvement factor, defined as

$$f_{improve} = C_{WT}/C \qquad (II)$$

expresses the amount of lipase variant protein needed to obtain the same effect as that obtained with 0.25 mg/l of the reference wild-type protein (C_{WT}).

Thus, the procedure for calculating the improvement factor was as follows:

1) The effect of the wild-type protein at 0.25 mg/l ($\Delta R_{wild-type}$) was calculated by means of equation (I);

2) the concentration of lipase variant resulting in the same effect as the wild-type at 0.25 mg/l was calculated by means of the following equation:

$$C = \left(K_{(variant)} \frac{\Delta R_{(wild\text{-}type)}}{\Delta R_{max(variant)} - \Delta R_{(wild\text{-}type)}}\right)^2 \qquad (III)$$

3) the improvement factor was calculated by means of equation (II).

The results are shown in Table 1 below.

Table 1

Variant	Improvement factor
D96K	4.0
D96W	2.7
D96F	1.7
D254K+L259I	1.7
D96W+D102N	3.4
L259I	1.2

It appears from Table 1 that the lipase variants D96K, D96W, D96W+E210N and to a certain extent the lipase variants D96F and D254K+L259I have a considerably better wash performance than the wild-type lipase. One possible explanation of this improved effect may be that the charge characteristic of the lipid contact zone of the variants have been changed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: DENMARK
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44448888
(H) TELEFAX: +45 4449 3256
(I) TELEX: 37304
(ii) TITLE OF INVENTION: Lipase Variants
(iii) NUMBER OF SEQUENCES: 2
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 918 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Humicola lanuginosa

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..873
(C) NAME/KEY: mat_peptide
(D) LOCATION: 67..873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG   48
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
    -20                 -15                 -10

GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC   96
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Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
-5 1 5 10

AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT 144
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
15 20 25

GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC 192
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
30 35 40

GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT 240
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
45 50 55

GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA 288
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
60 65 70

TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC 336
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75 80 85 90

GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC 384
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
95 100 105

TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT 432
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
110 115 120

ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT 480
Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
125 130 135

CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT 528
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
140 145 150

GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA 576
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155 160 165 170

TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC 624
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
175 180 185

GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT 672
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
190 195 200

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GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA  720
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT  768
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT  816
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG  864
Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

ACA TGT CTT TAGTGGCCGG CGCGGCTGGG TCCGACTCTA GCGAGCTCGA GATCT    918
Thr Cys Leu
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 291 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
    -20                 -15                 -10

Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
 -5                  1               5                  10

Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
                15                  20                  25

Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
            30                  35                  40

Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
        45                  50                  55
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Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
     60                  65                  70

Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
 75                  80                  85                  90

Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
                 95                 100                 105

Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
            110                 115                 120

Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
        125                 130                 135

Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
    140                 145                 150

Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
                175                 180                 185

Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
            190                 195                 200

Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

Thr Cys Leu
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CLAIMS

1. A lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule, in which a non-aromatic amino acid residue of a lipid contact zone comprising residues located within the part of the lipase structure containing the active serine residue, which residues may participate in the interaction with the substrate at or during hydrolysis, has been substituted with an aromatic amino acid residue.

2. A lipase variant according to claim 1, wherein the non-aromatic amino acid residue to be substituted is a glutamic acid or an aspartic acid residue.

3. A lipase variant according to claim 1 or 2, wherein the aromatic amino acid residue is selected from the group consisting of a tryptophan, a phenylalanine and a tyrosine residue.

4. A variant of a parent lipase, in which one or more amino acid residues of the mature *H. lanuginosa* lipase comprising the amino acid sequence shown in SEQ ID No. 1 are substituted as follows:

E56H, P, M, W, Y, F, I, G, C, V;
D96H, E, P, M, W, Y, F, I, G, C, V;
L259N, D, C, Q, E, H, I, M, F, P, W, Y;
L206K,R, N, D, C, Q, E, H, I, M, F, P, W, Y,

5. A lipase variant according to any of the preceding claims which comprises more than one substitution, preferably two substitutions.

6. A lipase variant of a parent lipase, in which one or more amino acid residues of the mature *H. lanuginosa* lipase comprising the amino acid sequence shown in SEQ ID No. 2 are substituted as follows:

D96W+E210N;
D254K+L259I;
D96L+L206V;
D96L+L206S;
D96W+D102N;
D96L+L259I+L206V;
E56Q+L259I+L206V,

7. A lipase variant according to any of the preceding claims, wherein the parent lipase is a microbial lipase.

8. A lipase variant according to claim 7, wherein the parent lipase is a fungal lipase.

9. A lipase variant according to claim 8, wherein the parent lipase is derived from a strain of Humicola, or Rhizomucor.

10. A lipase variant according to claim 9, wherein the parent lipase is a Rhizomucor miehei lipase.

11. A lipase variant according to claim 9, wherein the parent lipase is a H. lanuginosa lipase.

12. A lipase variant according to claim 8, wherein the parent lipase is a yeast lipase.

13. A lipase variant according to claim 12, wherein the parent lipase is derived from a strain of Candida.

14. A lipase variant according to claim 7, wherein the parent lipase is a bacterial lipase.

15. A lipase variant according to claim 14, wherein the parent lipase is derived from a strain of Pseudomonas.

16. A DNA construct comprising a DNA sequence encoding a lipase variant according to any of claims 1-15.

17. A recombinant expression vector which carries a DNA construct according to claim 16.

18. A cell which is transformed with a DNA construct according to claim 16 or a vector according to claim 17.

19. A cell according to claim 18 which is a fungal cell, e.g. belonging to the genus Aspergillus, such as A. niger, A. oryzae, or A. nidulans; a yeast cell, e.g. belonging to a strain of Saccharomyces, such as S. cerevisiae, or a methylotrophic yeast from the genera Hansenula, such as H. polymorpha, or Phichia, such as P. pastoris; or a bacterial cell, e.g. belonging to a strain of Bacillus, such as B. subtilis, or B. lentus.

20. A method of producing a lipase variant according to any of claims 1-15, wherein a cell according to claim 18 or 19 is cultured under conditions conducive to the production of the lipase variant, and the lipase variant is subsequently recovered from the culture.

21. A detergent additive comprising a lipase variant according to any of claims 1-15, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

22. A detergent additive according to claim 21 which contains 0.02-200 mg of enzyme protein/g of the additive.

23. A detergent additive according to claim 21 or 22 which additionally comprises another enzyme such as a protease, amylase, oxidase, peroxidase, cellulase and/or a lipase different from the lipase variant.

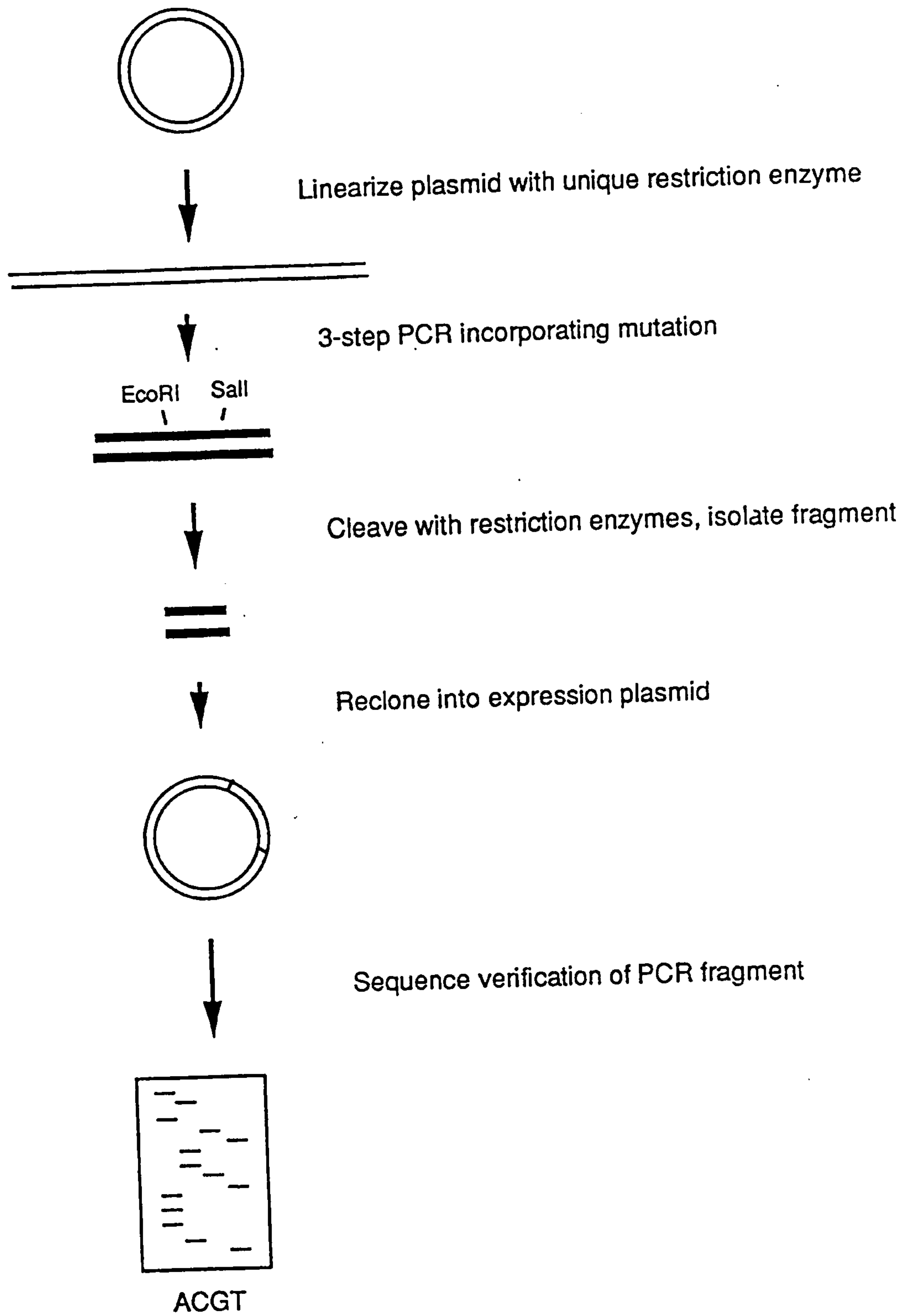
24. A detergent composition comprising a lipase variant according to any of claims 1-15.

25. A detergent composition according to claim 24 which additionally comprises another enzyme such as a protease, amylase,

oxidase, peroxidase, cellulase, and/or a lipase different from the lipase variant.

26. A diswashing detergent composition comprising a lipase variant according to any of claims 1-15.

27. A softening composition comprising a lipase variant according to any of claims 1-15.



Linearize plasmid with unique restriction enzyme
3-step PCR incorporating mutation
EcoRI
SalI
Cleave with restriction enzymes, isolate fragment
Reclone into expression plasmid
Sequence verification of PCR fragment
ACGT

Fig. 1

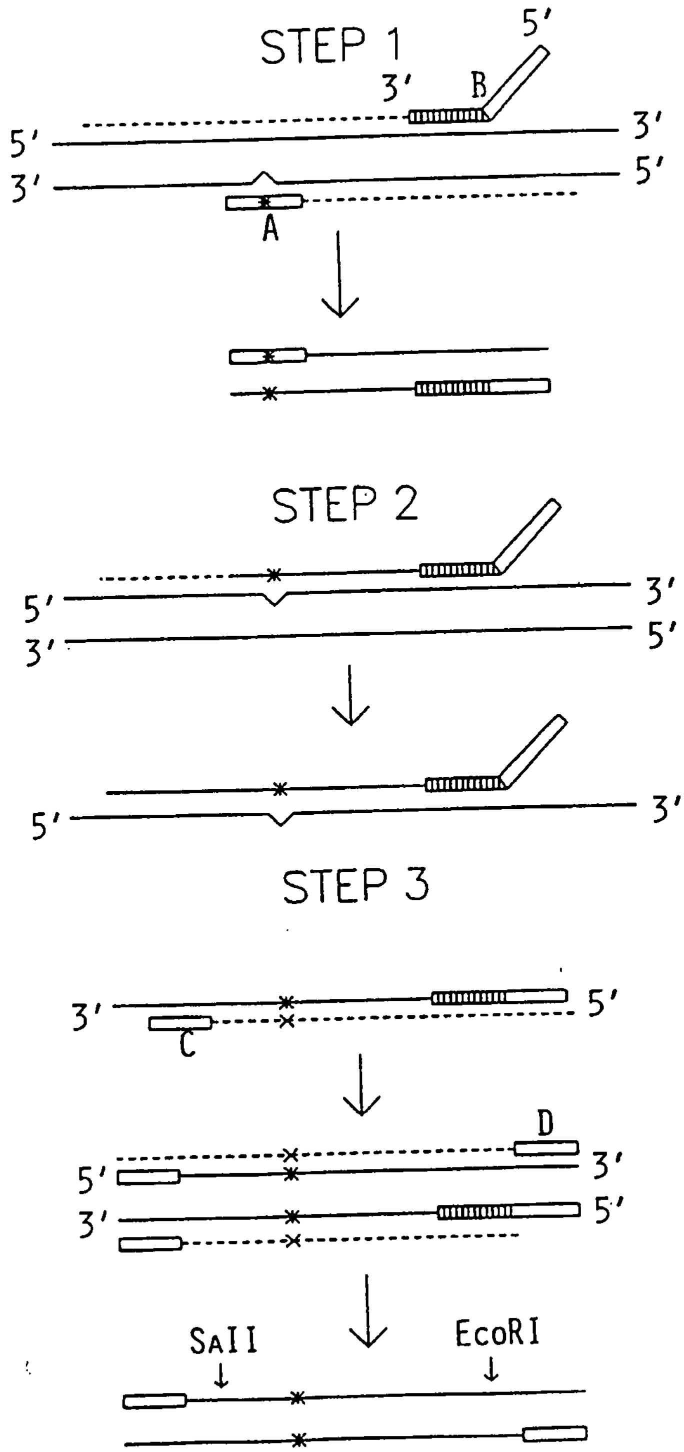


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00162

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/20, C11D 3/386, C12N 15/55
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, BIOSIS, MEDLINE, BIOTECHNICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A1, 0305216 (NOVO INDUSTRI A/S), 1 March 1989 (01.03.89)	1-17
X		18-20
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A	EP, A1, 0407225 (UNILEVER PLC), 9 January 1991 (09.01.91)	1-25
	--	
X	WO, A1, 9205249 (NOVO NORDISK A/S), 2 April 1992 (02.04.92), see claims	1-25
	--	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" erlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1994	29 -07- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/07/94

International application No.
PCT/DK 94/00162

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0305216	01/03/89	JP-A- 1157383	20/06/89
		JP-C- 1761424	20/05/93
		JP-B- 4038394	24/06/92
EP-A1- 0407225	09/01/91	JP-T- 4500608	06/02/92
		WO-A- 9100910	24/01/91
WO-A1- 9205249	02/04/92	AU-A- 8617291	15/04/92
		CA-A- 2092615	14/03/92
		EP-A- 0548228	30/06/93
		JP-T- 6501153	10/02/94